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Author(s): Richard K. Gast, Jean Guard-Petter, Peter S. Holt
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Richard K. Gast, Jean Guard-Petter, and Peter S. Holt

United States Department of Agriculture, Agricultural Research Service,
Southeast Poultry Research Laboratory, 934 College Station Road, Athens, GA 30605

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SUMMARY. Experimental infection models are valuable tools for understanding and preventing the deposition of *Salmonella enteritidis* inside eggs. Oral inoculation is believed to closely simulate naturally occurring *S. enteritidis* infections of chickens, but oral infection studies have often generated relatively low frequencies of egg contamination. The present study assessed whether repeated *in vivo* passage of an *S. enteritidis* strain could affect its ability to cause egg contamination in experimentally infected hens. The incidence of egg contamination was determined in groups of hens inoculated orally with either a phage type 13a *S. enteritidis* strain or derivatives of this parent strain that were obtained by three successive rounds of passage and reisolation from tissues of infected hens. Passaged *S. enteritidis* isolates recovered from ovaries and oviducts induced a significantly higher incidence of egg contamination (16.97%) than was attributed to the parent strain (8.27%). However, passaged *S. enteritidis* isolates recovered from livers and spleens were not associated with a significantly increased frequency of deposition in eggs. By either inducing or selecting for the expression of relevant microbial properties, passage of *S. enteritidis* through reproductive tissues of chickens may be useful for improving the efficiency at which experimental infection models produce egg contamination.

RESUMEN. Efecto de pasajes seriados in vivo en la frecuencia de contaminación con Salmonella enteritidis de huevos de ponedoras infectadas experimentalmente.

Los modelos de infección experimental son una herramienta valiosa para entender y prevenir la deposición de *Salmonella enteritidis* en el interior de los huevos. Se cree que la inoculación por vía oral muestra una similitud cercana a las infecciones naturales de los pollos por *S. enteritidis*, sin embargo, estudios de infecciones orales han generado con frecuencia niveles relativamente bajos de contaminación de los huevos. En el presente estudio se evalúa si pasajes repetidos *in vivo* de una cepa de *S. enteritidis* afectarían su capacidad para contaminar los huevos de ponedoras infectadas experimentalmente. Se determinó la incidencia de contaminación de los huevos en grupos de ponedoras inoculadas por vía oral con una cepa de *S. enteritidis* fagotipo 13a o con sus cepas derivadas, las cuales fueron obtenidas mediante tres rondas sucesivas de pasaje-reaislamiento a partir de tejidos de ponedoras infectadas. Los aislamientos pasados de *S. enteritidis*, reaislados a partir de ovarios y oviductos, indujeron un mucho mayor incremento significativo en la contaminación de los huevos (16.97%) que el atribuido a la cepa original (8.27%). Sin embargo, los aislamientos pasados de *S. enteritidis*, reaislados a partir de hígados y bazos, no estuvieron asociados con un incremento significativo de la frecuencia de deposición en los huevos. Mediante la inducción o selección de la expresión de propiedades microbiológicas relevantes, el pasaje de *S. enteritidis* a través del tejido reproductor de pollos puede ser útil para mejorar la eficiencia con la cual los modelos de infecciones experimentales producen contaminación de los huevos.

Key words: *Salmonella enteritidis*, chickens, egg contamination, *in vivo* passage

Abbreviations: BG = brilliant green; L/S = liver/spleen; O/O = ovary/oviduct; TS = tryptone soya

Eggs contaminated internally with *Salmonella enteritidis* are epidemiologically significant sources of foodborne human illness in the United States and elsewhere (1,8). Control of *S. enteritidis* infections in laying flocks has accordingly become a cornerstone of most proposed strategies for reducing the

risk of egg-associated transmission of disease (23,34). Both naturally and experimentally infected hens can deposit this pathogen inside eggs (12,16,25,26). The production of contaminated eggs by experimentally infected chickens has several important applications for evaluating potential disease control options. Experimentally induced egg contamination can be used to determine the predictive relevance of other measurable parameters (such as fecal shedding or the antibody response) for detecting *S. enteritidis* infections in laying flocks, to characterize qualitative and quantitative features of *S. enteritidis* deposition in eggs in order to develop effective egg refrigeration standards, and to assess the efficacy of preventive practices such as vaccination. However, the usefulness of experimental infection models for these purposes is heavily dependent on the actual frequency of egg contamination that they generate.

The observed frequency of isolation of *S. enteritidis* from eggs laid by naturally infected commercial flocks has been less than 0.03% in most surveys (28,35). The overall incidence of *S. enteritidis* in eggs in the United States has been estimated at only 0.005% (11). Relatively low egg contamination frequencies have also been reported in most experimental infection studies, even after the administration of extremely large oral doses of *S. enteritidis* (15,16,17). Alternative routes of exposure to *S. enteritidis*, including intravenous and aerosol administration, have likewise usually led to rather infrequent production of contaminated eggs (3,15, 29,32,33). Efficient detection of egg contamination is further complicated by the small initial number of *S. enteritidis* cells typically found in freshly laid eggs from either naturally or experimentally infected hens (13,16,25,26). Moreover, laboratory passage and storage of *S. enteritidis* isolates may attenuate their ability to cause egg contamination. For example, a phage type 13a *S. enteritidis* strain that produced egg contamination frequencies of 8%–16% in three oral infection studies conducted between 1990 and 1992 (12,13,14) was associated with egg contamination frequencies of only 3%–5% in four similar studies conducted between 2000 and 2002 (15,16,17,18).

Egg contamination by *S. enteritidis* appears to result from the colonization of reproductive tissues, especially the ovary and upper oviduct, in systemically infected chickens (27,32,33). The location (yolk or albumen) of *S. enteritidis* deposition in a developing egg is likely a consequence of which regions of the laying hen's reproductive tract are colonized (7,16,25,26). Therefore, the potential for experimental infection models to generate internally contaminated eggs may depend on the extent to which they lead to *S. enteritidis* colonization of the ovary and oviduct. Selecting for (or inducing) the ability of *S. enteritidis* strains to colonize reproductive tissues of poultry could thus improve the frequency of egg contamination obtained by experimental infection. The objective of the present study was to determine whether repeated *in vivo* passage of a phage type 13a *S. enteritidis* strain in chickens would affect its subsequent frequency of deposition inside eggs laid by experimentally infected hens.

MATERIALS AND METHODS

Laying hens. Two replicate trials were conducted, each of which involved a series of *in vivo* passages of an *S. enteritidis* isolate through laying hens followed by an experimental infection experiment to determine the effect of these passages on the frequency of egg contamination caused by this isolate. In the passage phase of each trial, 30 laying hens obtained from our laboratory's specific-pathogen-free flock of single-comb white leghorn chickens were distributed evenly among three groups in separate rooms of a disease-containment facility. The hens (25 and 35 wk old at the beginning of the first and second trials, respectively) were housed individually in laying cages and provided with water and pelleted feed *ad libitum.* In the egg contamination phase of each trial, 60 laying hens (35 and 48 wk old at the beginning of the first and second trials, respectively) were distributed evenly among three separately housed groups.

Preparation of *S. enteritidis* cultures. At the beginning of the passage phase in each trial, frozen storage beads containing a phage type 13a strain of *S. enteritidis* were resuscitated by incubation for 24 hr at 37 C in tryptone soya (TS) broth (Oxoid Limited, Basingstoke, Hampshire, England) and subsequent transfer by streaking onto plates of brilliant green (BG) agar (Becton, Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO). After incubation of these plates for 24 hr at 37 C, four typical *S. enteritidis* colonies were transferred to TS broth and incubated at 37 C for 24 hr. Each hen was inoculated with a 1-ml dose of this culture containing approximately 2.0×10^9 colony-forming units of *S. enteritidis*. TS broth cultures for inoculation of hens in the subsequent rounds of infection in each trial were prepared by combining two typical *S. enteritidis* colonies from BG agar plates derived from two different birds in a prior round as described below for each passage.

Serial *in vivo* passage of *S. enteritidis* in laying hens. Prior to the inoculation of groups of

hens to determine the resulting frequency of egg contamination, the parent *S. enteritidis* strain was subjected to three successive rounds of *in vivo* passage in each trial. For passage 1, each member of one group of 10 hens was inoculated orally with the *S. enteritidis* parent strain. Seven days later, these hens were euthanatized for the removal and bacteriologic culturing of internal organ samples. Portions of the liver and spleen from each bird were pooled together (and designated the L/S sample), as were portions of the ovary and oviduct from each bird (designated the O/O sample). Each organ pool was transferred to 50 ml of tetrathionate BG broth (Oxoid), mixed by stomaching for 30 sec, and incubated for 24 hr at 37 C. Each sample was then streaked onto BG agar and incubated for 24 hr at 37 C. The identity of prospective *S. enteritidis* colonies was confirmed biochemically and serologically (38).

For passage 2, a second group of 10 hens was orally infected with *S. enteritidis* isolates obtained from passage 1. Five hens received a mixture of L/S isolates from two different birds in passage 1, and five hens received a corresponding mixture of two O/O isolates. At 7 days postinoculation, these hens were euthanatized for the bacteriologic culturing of tissue samples (L/S and O/O) as described above. For passage 3, the remaining group of 10 hens was orally infected with *S. enteritidis* isolates obtained from passage 2. Five hens were given a mixture of L/S isolates from two different birds in passage 2 (both of which had themselves received L/S isolates from passage 1), and five hens were given a corresponding mixture of two O/O isolates. At 7 days postinoculation, these hens were euthanatized for the collection and culturing of organ samples (L/S and O/O) as described above.

Egg contamination after infection with passaged *S. enteritidis* isolates. In each of the two replicate trials, one group of 20 hens was infected orally with the *S. enteritidis* parent strain. A second group of 20 hens was inoculated with a mixture of L/S isolates from two different birds in passage 3 (both of which had themselves received L/S isolates from passage 2), and the remaining group of 20 hens was inoculated with a mixture of O/O isolates from two different birds in passage 3 (both of which had themselves received O/O isolates from passage 2). Samples of voided feces were collected from each hen and cultured for the presence of *S. enteritidis* by previously described methods (19) immediately before inoculation and at 1, 2, and 3 wk postinoculation.

All eggs laid by each hen between 1 and 24 days postinoculation were collected and cultured to detect internal contamination with *S. enteritidis*. Eggshell surfaces were disinfected by dipping for 5 sec in 70% ethanol, and the shells were broken against sterile foil strips. The entire liquid content of each egg was transferred to 50 ml of TS broth supplemented with 100 mg/l of ferrous sulfate (Sigma), mixed by vigorous shaking for 15 sec, and incubated for 24 hr at 37 C. A 1-ml portion of the incubated TS broth culture was then transferred to 9 ml of Rappaport Vassiliadis broth (Oxoid) and incubated for 24 hr at 37 C. Each sample was then streaked onto plates of BG agar and again incubated for 24 hr at 37 C, followed by biochemical and serologic confirmation of the identity of presumptive colonies of *S. enteritidis*.

Statistical analysis. For each replicate egg contamination trial, significant differences ($P < 0.05$) among inoculation groups (parent, L/S, and O/O) in the mean frequency of recovery of *S. enteritidis* from voided feces or egg contents were determined by Kruskal–Wallis analysis of variance followed by Dunn multiple comparison test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

RESULTS

Detection of *S. enteritidis* in fecal samples. All fecal samples collected before inoculation were negative for *Salmonella*. For both trials combined, *S. enteritidis* was recovered from the feces of 95% of hens in each of the three treatment groups at 1 wk postinoculation (Fig. 1). By 2 wk postinoculation, fecal shedding of *S. enteritidis* had declined to 55%–60% in all groups. At 3 wk postinoculation, 35% of hens inoculated with the L/S-passaged isolate, 32.5% of hens inoculated with the parent strain, and only 12.5% of hens inoculated with the O/O-passaged isolate yielded fecal samples that were positive for *S. enteritidis*. Nevertheless, the frequencies of isolation of *S. enteritidis* from feces in the three treatment groups were not significantly different.

Detection of *S. enteritidis* in egg content samples. No significant differences in total egg production were observed among the three treatment groups. For both trials combined, hens inoculated with the parent strain laid 629 eggs, hens inoculated with the L/S-passaged strain laid 653 eggs, and hens inoculated with the O/O-passaged isolate laid 707 eggs. During the sampling interval from 1 to 24 days postinoculation, *S. enteritidis* was isolated from the contents of eggs laid by hens in all three treatment groups (Fig. 2). For both trials combined, hens that received the parent strain produced contaminated eggs between 7 and 23 days postinoculation (with a peak incidence at 12 days), hens that received the L/S-passaged isolate laid contaminated eggs between 6 and 23 days postinoculation (with a peak incidence at 12 days), and hens that received the O/O-passaged isolate laid

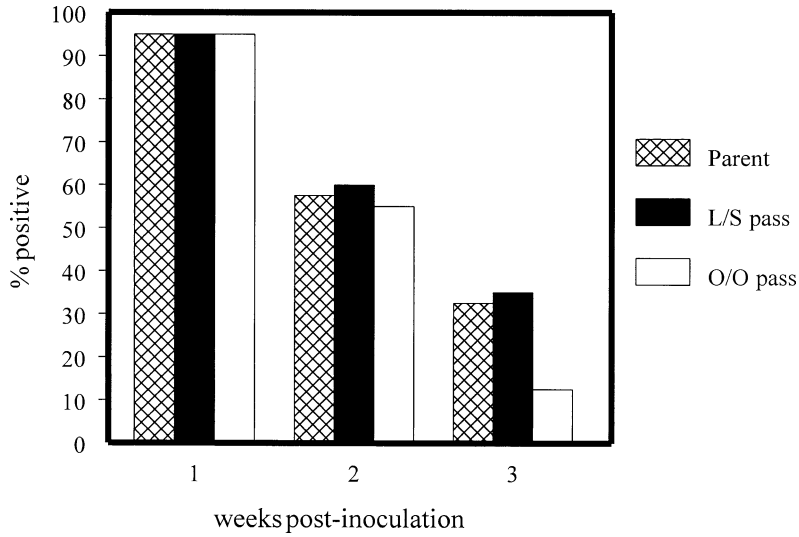

Fig. 1. Frequency of recovery of *Salmonella enteritidis* from samples of voided feces after inoculation of laying hens ($n = 40$ per treatment group) with three isolates: a phage type 13a parent strain, a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from livers and spleens (L/S pass), or a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from ovaries and oviducts (O/O pass).

contaminated eggs between 6 and 24 days postinoculation (with a peak incidence at 11 days).

The relative frequencies of deposition of *S. enteritidis* inside eggs laid by the three treatment groups of hens were very similar in the two trials (Fig. 3). For both trials combined, 16.97% of eggs from hens inoculated with the O/O-passaged isolate, 10.41% of eggs from hens inoculated with the L/S-passaged isolate, and 8.27% of eggs from hens inoculated with the parent strain were contaminated with *S. enteritidis*. The overall frequency of egg contamination associated with the O/O-passaged isolate was significantly higher than for either the L/S-passaged isolate ($P < 0.05$) or the parent strain ($P < 0.005$). However, the frequency of recovery of *S. enteritidis* from egg contents was not significantly greater for the L/S-passaged isolate than for the parent strain.

DISCUSSION

In the present study, prior serial passage of a phage type 13a isolate of *S. enteritidis* through ovaries and oviducts of laying hens led to a significant increase in the frequency of deposition of this strain in eggs during subsequent oral infection trials. However, serial passage through livers and spleens did not significantly affect the ability of this strain to cause egg contamination. The colonization of reproductive tissues is often identified as a pivotal step in the sequence of events that leads to the deposition of *S. enteritidis* inside eggs (27,32,33). However, low frequencies of egg contamination by *S. enteritidis* have sometimes been reported from hens with relatively high incidences of ovarian colonization (2,31). Therefore, some microbial property that becomes relevant after colonization of reproductive organs, in addition to the ability to reach and colonize these organs, may be necessary for *S. enteritidis* deposition in egg contents.

The low frequency of isolation of the O/O-passaged isolate from fecal samples at 3 wk postinoculation in the present study could be the consequence of a diminished ability to persist in the intestinal tract. Intestinal colonization by *Salmonella pullorum*, a vertically transmissible poultry pathogen, is likewise often reported to be more transient than is typical for paratyphoid *Salmonella* serotypes (36). Persistent intestinal colonization and fecal shedding by *S. enteritidis* have not been particularly consistent indicators of the probability of systemic infection and egg contamination (14,16,24).

The outcomes of *S. enteritidis* infection in poultry are influenced by host susceptibility characteristics as well as microbial factors. Susceptibility to intestinal colonization, organ invasion, and egg deposition have all been reported to vary between lines of chickens (4,5,6). Accordingly, comparisons between experimental infection studies that used

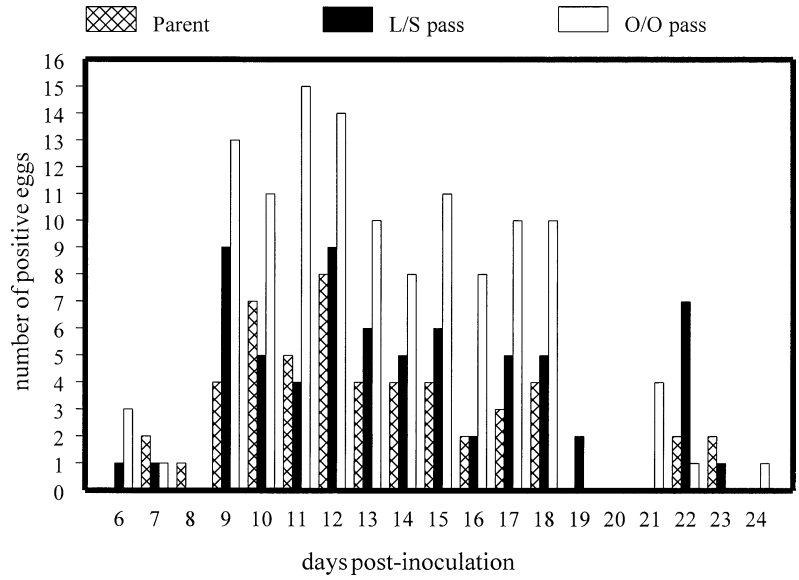

Fig. 2. Daily numbers of internally contaminated eggs produced after inoculation of laying hens ($n = 40$ per treatment group) with three *Salmonella enteritidis* isolates: a phage type 13a parent strain, a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from livers and spleens (L/S pass), or a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from ovaries and oviducts (O/O pass).

different lines of hens may not be entirely dependable. However, such considerations were not applicable within the current study, which used chickens from a highly inbred population.

The present results suggest that the interaction of *S. enteritidis* with reproductive tissues of chickens may have either induced or selected for the expression of microbial properties responsible for egg

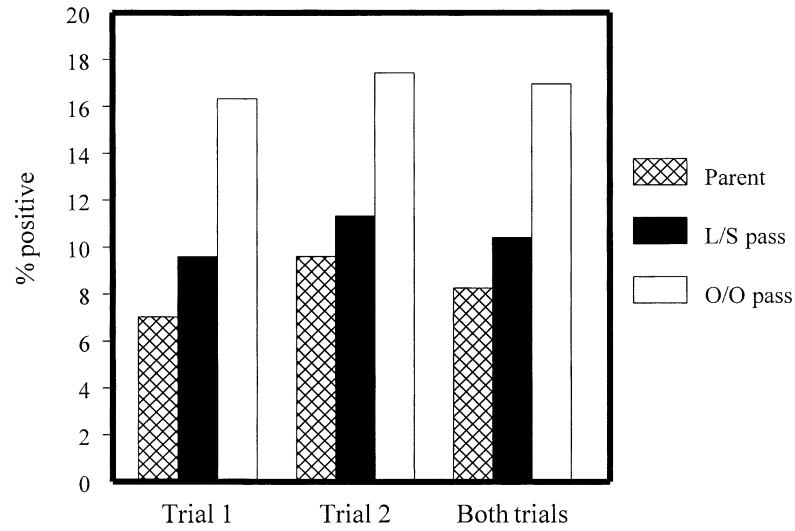

Fig. 3. Frequency of recovery of *Salmonella enteritidis* from internal contents of eggs laid during the first 24 days after inoculation of laying hens ($n = 20$ per treatment group in each trial) with three isolates: a phage type 13a parent strain, a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from livers and spleens (L/S pass), or a derivative of the parent strain obtained by repeated *in vivo* passage and reisolation from ovaries and oviducts (O/O pass).

contamination. Environmental conditions such as pH and temperature have been shown previously to influence the expression of potential virulence factors such as flagella, fimbria, and outer membrane proteins by *S. enteritidis* (10,30,37). Growth of *S. enteritidis* in chicken tissues has been reported similarly to affect the expression of flagella, fimbria, and iron uptake systems (9). The ability to cause infected hens to lay contaminated eggs can vary significantly between *S. enteritidis* strains (16,18). Diverse phenotypic properties of *S. enteritidis* isolates, including growth to high cell densities (22) and expression of high-molecular-mass lipopolysaccharide (20), have been linked to egg contamination. The complementarity of multiple microbial attributes may be important in influencing the likelihood of egg contamination (21). For example, a mixture of *S. enteritidis* strains expressing both attributes related to invasion beyond the intestinal tract and attributes related to colonization of reproductive tissues has been used successfully to promote egg contamination in prior experimental infection studies (15).

The production of contaminated eggs is critical to the usefulness of experimental infection models for developing and evaluating *S. enteritidis* control strategies. The current experiments demonstrated that repeated passage through reproductive tissues of chickens increased the ability of an *S. enteritidis* strain to induce internal contamination of eggs in an oral infection model. Further characterization of the genetic basis for the observed effects of *in vivo* passage should be valuable both for refining the efficiency of experimental infection models in generating egg contamination and for understanding the mechanisms responsible for *S. enteritidis* deposition inside eggs.

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